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Report

Antiproliferative activity *in vitro* of new malatoplatinum(II) complexes

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The results of studies on antiproliferative activity *in vitro* of nine new platinum(II) complexes against cells of eight human and six murine neoplastic cell lines are described. New complexes with the anionic rest originating from enantiomeric forms of hydroxydicarboxylic malic acid were synthesized to obtain agents with increased water solubility and decreased toxicity. Three compounds, coded 1–3, with ethylenediamine as a neutral ligand, showed cytotoxic activity against 12 out of 14 target cell lines. Their cytotoxic activity was similar or even slightly higher than that of the reference carboplatin. The remaining six compounds, coded 4–9, with 1-alkylimidazole as a neutral ligand, revealed rather low cytotoxic activity, and only against the cells of the human bladder cancer cell line Hu1703He, ovarian cancer cell line OAW-42 and mouse leukemia P388. Most of them appeared to be negative against all other cell lines. No compounds, including reference carboplatin, showed any cytotoxicity against the cells of the T47D human breast cancer cell line or B16F-10 mouse melanoma cell line. The results obtained are in accordance with common opinion, i.e. that the presence of neutral amine ligands with NH groups is required for the cytotoxic activity of platinum complexes. Compounds with a primary amine (ethylenediamine) showed higher cytotoxic activity *in vitro* than complexes with a tertiary amine (1-alkylimidazole). [© 2000 Lippincott Williams & Wilkins.]

Key words: Antitumor activity *in vitro*, carboplatin, human cancer cell lines, malatoplatinum(II) complexes, platinum cytostatics.

Introduction

In the search for new analogs of cisplatin and carboplatin, dicarboxylate platinum complexes belong to the most successfully examined agents. Their pharmacological properties ensure stability in aqueous solutions and, subsequently, their increased resistance to substitution reactions with potential ligands during their transport to the target molecule. Despite their low degree of hydrolysis, dicarboxylate complexes are effective in binding DNA.[1] Possibly, such compounds should reveal low toxicity but still retain sufficient cytotoxicity to cancer cells.

In our search for low toxicity platinum anticancer agents, the platinum(II) complexes with the anionic rest originating from hydroxydicarboxylic acids, such as L(−), D(+) or DL-malic acid, were synthesized. We hypothesized that hydroxyl function in anionic ligands will increase the water solubility and will reduce the lipophilicity of the compound. The latter seems to be correlated with side effects, as central and peripheral neuropathy is observed in patients treated with conventional platinum drugs.[2] Moreover, use of the enantiomeric complexes, whose chirality is caused by the stereochemistry of malate ligand, could explain the eventual role of the dicarboxylate anion in an active form of the platinum complex. Considering that the final form reacting with DNA contains no remaining ligands, e.g. $[PtA_2(OH)(H_2O)^+]$, similarly to the cisplatin-reactive form, one can expect that all three complexes, differing only in the malate anion by the D, L or DL configuration should have a similar cytotoxic activity. However, if some differences in their cytotoxicity are observed, it is reasonable to expect that the reactive forms contain dicarboxylate ligands attached to the platinum center, since chiral biological targets can only distinguish substrates of this form.

The investigated complexes also differ in their

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neutral ligands: ethylenediamine or *N*-ethylimidazole or *N*-propylimidazole. Complexes with ethylenediamine belong to the classical group of platinum analogs, because the neutral ligand contains protons at the *N*-atom donor supposed to be necessary for effective binding with DNA.[3] The complexes containing *N*-alkylimidazole do not fulfill this requirement and, in general, they may be less cytotoxic than conventional analogs. However, we expected to find among them the compound(s) which would react with target DNA in another way than conventional drugs and, consequently, would exhibit another spectrum of activity including overcoming resistance of cancer cells to cisplatin.

The aim of the present study was to evaluate the antiproliferative *in vitro* activity of nine new malatoplatinum(II) complexes and to compare it with the activity of carboplatin used as referential agent.

Materials and methods

Compounds

The following compounds were examined in the *in vitro* screening assay (Figure 1):
1, $Pt(C_2H_8N_2)(D\text{-}C_4H_4O_5)$; **2**, $Pt(C_2H_8N_2)(DL\text{-}C_4H_4O_5)$; **3**, $Pt(C_2H_8N_2)(L\text{-}C_4H_4O_5)$; **4**, $Pt(C_5H_8N_2)_2(DL\text{-}C_4H_4O_5)$; **5**, $Pt(C_5H_8N_2)_2(D\text{-}C_4H_4O_5)$; **6**, $Pt(C_5H_8N_2)_2(L\text{-}C_4H_4O_5)$; **7**, $Pt(C_6H_{10}N_2)_2(D\text{-}C_4H_4O_5)$; **8**, $Pt(C_6H_{10}N_2)_2(DL\text{-}C_4H_4O_5)$; **9**, $Pt(C_6H_{10}N_2)_2(L\text{-}C_4H_4O_5)$. The synthesis, purification and identification as well as the stability in aqueous solution and susceptibility towards substitution of these malatoplatinum(II) complexes were described earlier.[4] All complexes are stable in aqueous solution and reveal low reactivity with potential biological ligands.[4] Carboplatin was purchased from Sigma-Aldrich (St Louis, MO).

Test solutions of the compounds tested (1 mg/ml) were prepared by dissolving the substance in 100 μl of water for injections completed with 900 μl of tissue culture medium. Afterwards, the tested compounds were diluted in culture medium (described below) to reach the final concentrations of 100, 10, 1 and 0.1 μg/ml.

Cells

The following established *in vitro* human cancer cell lines were used: SW707 (rectal adenocarcinoma), A549 (non-small cell lung carcinoma), T47D and MCF-7 (breast cancers), and SKOV-3 and OAW-42 (ovarian cancers). All lines were obtained from the ATCC (Rockville, MD) and are maintained in the Cell Culture Collection of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. Human uroepithelial cell lines Hu1703He and HCV29T, established in the Fibiger Institute (Copenhagen, Denmark), were obtained from Dr J Kieler in 1982 and maintained at the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

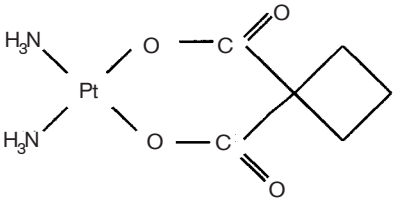
The following established *in vitro* murine cancer cell lines were used: 16/C (mammary cancer), B16F-10 (melanoma), C38 (colon cancer), LL_2 (lung cancer), and P388 and L1210 (leukemia). The cells are maintained in the Cell Culture Collection of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

Twenty-four hours before addition of the tested agents, the cells were plated in 96-well plates (Sarstedt, Newton, NC) at a density of 10^4 cells/well. The cells were cultured in opti-MEM medium supplemented with 2 mM glutamine (Gibco, Warsaw, Poland), streptomycin (50 μg/ml), penicillin (50 U/ml) (both antibiotics from Polfa, Tarchomin, Poland) and 5% fetal calf serum (Gibco, Grand Island, NY). The cell cultures were maintained at 37°C in humid atmosphere saturated with 5% CO_2.

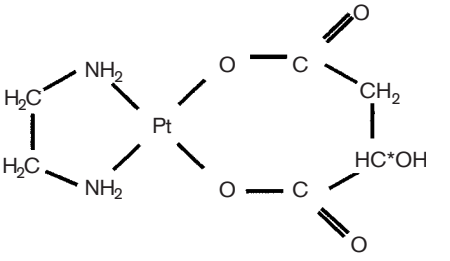
Anti-proliferative assay *in vitro*

Sulforhodamine B (SRB). Details of this technique were described by Skehan *et al.*[5] The cytotoxicity assay was performed after 72 h exposure of the cultured cells to varying concentrations (from 0.1 to 100 μg/ml) of the tested agents. The cells attached to the plastic were fixed by gently layering cold 50% (trichloroacetic acid (TCA; Aldrich-Chemie, LOCATION??, Germany) on the top of the culture medium in each well. The plates were incubated at 4°C for 1 h and then washed 5 times with tap water. The background optical density was measured in the wells filled with culture medium, without the cells. The cellular material fixed with TCA was stained with 0.4% SRB (Sigma, Steinheim, Germany) dissolved in 1% acetic acid (POCh, Gliwice, Poland) for 30 min. Unbound dye was removed by rinsing (4 times) with 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base (POCh) for determination of optical density (at 540 nm) in a computer-interfaced, 96-well microtiter plate reader Multiskan RC photometer (Labsystems, Helsinki, Finland). Each compound at given concentrations was tested in triplicates in each experiment, which was repeated 3–5 times.

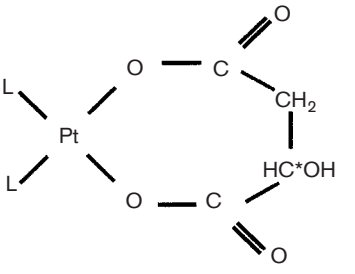
MTT. This technique was applied for cytotoxicity screening against mouse leukemia cells growing in



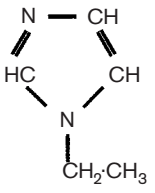
Carboplatin
(MW 371.3)



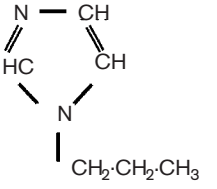
Ethylenediaminemalatoplatinum (II),**1 – 3**
(MW 387.3)



L = **a:** bis(1-ethylimidazole)malatoplatinum(II), **4 – 6**
(MW 519.4)
L = **b:** bis(1-propylimidazole)malatoplatinuim(II), **7 – 9**
(MW 547.5)



a: L = 1-ethylimidazole



b: L = 1-propylimidazole

Figure 1. The structures of platinum complexes investigated. C*, chiral center.

suspension culture. An assay was performed after 72 h exposure to varying concentrations (from 0.1 to 100 μg/ml) of the tested agents. For the last 3–4 h of incubation 20 μl of MTT solution was added to each well [MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma); stock solution: 5 mg/ml]. The mitochondria of viable cells reduce a pale yellow MTT to a navy blue formazan, so if more viable cells are present in the well, more MTT will be reduced to formazan. When the incubation time was completed, 80 μl of the lysing mixture was added to each well [lysing mixture: 225 ml dimethylformamide, 67.5 g sodium dodecylsulfate (both from Sigma) and 275 ml of distilled water]. After 24 h, when formazan crystals had been dissolved, the optical densities of the samples were read on an Multiskan RC photometer (Labsystems) at 570 nm wavelength.

Results and discussion

The results of cytotoxic activity *in vitro* were expressed as ID_{50}, i.e. the dose of compound (in μM) that inhibits the proliferation rate of the tumor

cells by 50% as compared to control untreated cells.

As shown in Table 1, only three compounds, **1–3**, revealed cytotoxic activity against seven out of eight human target cell lines. The ID_{50} values of these compounds varied from 4.1 to 95.5 μM and were similar or lower than ID_{50} values for carboplatin (12.7–210.0 μM). The remaining compounds, **4–9**, revealed cytotoxic activity only against cells of the OAW-42 and Hu1703He lines (Figure 2), with ID_{50} values varying from 57.2 to 144.0 μM, slightly higher than the ID_{50} for carboplatin (12.7 and 99.6 μM, respectively). In addition, compound **4** revealed antiproliferative activity against cells of the SKOV-3 and MCF-7 cell lines and compounds **7–9** against MCF-7 cells (Table 1). No compounds, including reference carboplatin, were cytotoxic to the cells of T47D human breast cancer cell line. It is known from clinical studies that breast cancers are generally intrinsically unresponsive to platinum-based chemotherapy.[6] Interestingly, the cells of OAW-42 (human ovarian cancer cell line) appeared

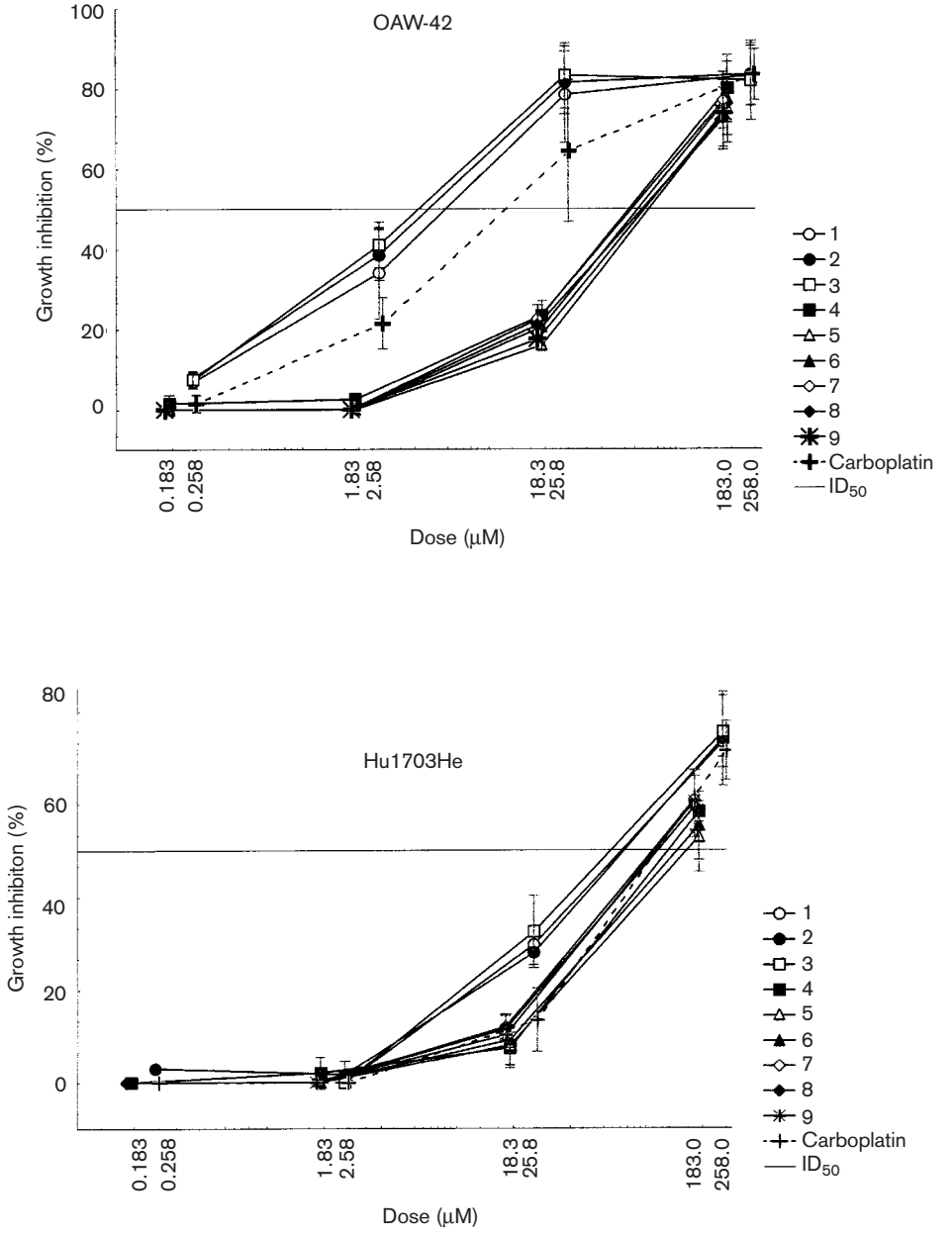


Figure 2. Antiproliferative activity *in vitro* of new malatoplatinum(II) complexes against the cells of OAW-42 human ovarian cancer cell line and Hu1703He human bladder cancer cell line.

Table 1. The antiproliferative activity *in vitro* of nine new malatoplatinum(II) complexes (expressed as ID_{50}) against human cancer cell lines

Compound		Cell line/ID_{50} (μM)						
No.	Formula	A549	SW707	Hu1703	HCV29T	SKOV-3	OAW-42	MCF-7
1	$Pt(C_2H_8N_2)$(D-$C_4H_4O_5$)	85.2[a]	80.0	74.9	23.2	64.3	5.4	85.7
2	$Pt(C_2H_8N_2)$(DL-$C_4H_4O_5$)	87.8	95.5	80.0	31.0	53.7	4.7	77.7
3	$Pt(C_2H_8N_2)$(L-$C_4H_4O_5$)	78.8	77.5	65.8	18.1	59.6	4.1	75.1
4	$Pt(C_5H_8N_2)_2$(DL-$C_4H_4O_5$)	–[b]	–	125.0	–	147	57.2	88.4
5	$Pt(C_5H_8N_2)_2$(D-$C_4H_4O_5$)	–	–	138.0	–	–	73.7	–
6	$Pt(C_5H_8N_2)_2$(L-$C_4H_4O_5$)	–	–	144.0	–	–	63.3	–
7	$Pt(C_6H_{10}N_2)_2$(D-$C_4H_4O_5$)	–	–	103.0	–	–	58.6	112.0
8	$Pt(C_6H_{10}N_2)_2$(DL-$C_4H_4O_5$)	–	–	111.0	–	–	67.2	136.0
9	$Pt(C_6H_{10}N_2)_2$(L-$C_4H_4O_5$)	–	–	111.0	–	–	69.6	137.0
Carboplatin		108.0	210.0	99.6	68.7	86.5	12.7	139.0

[a]Standard deviations varied from 0.2 to 9.9 μM.
[b]negative.

Table 2. The antiproliferative activity *in vitro* of new malatoplatinum(II) complexes (expressed as ID_{50}) against mouse neoplastic cell lines

Compound		Cell line/ID_{50} (μM)				
No.	Formula	C38	LL_2	16/C	L1210	P388
1	$Pt(C_2H_8N_2)$(D-$C_4H_4O_5$)	62.0[a]	108.0	91.7	77.7	7.9
2	$Pt(C_2H_8N_2)$(DL-$C_4H_4O_5$)	66.4	98.1	101.0	79.5	5.5
3	$Pt(C_2H_8N_2)$(L-$C_4H_4O_5$)	66.4	98.1	101.0	67.6	5.4
Carboplatin		87.3	141.0	151.0	71.9	10.4

[a]Standard deviations varied from 2.6 to 5.2 μM.

to be the most sensitive target among all human cell lines tested (Table 1 and Figure 2). These results seem to correlate with clinical experience indicating that carboplatin is the effective drug in the treatment of ovarian cancer patients.[7] On the other hand, the cells of the SKOV-3 ovarian cancer cell line (known to be rather resistant to platinum compounds[8]) appeared to be less sensitive in our assay than OAW-42 cells. This, to a certain degree, may reflect a very common clinical situation concerning individual differences in the response to anticancer chemotherapy between patients with a similar type of ovarian cancer. An identical situation was observed in the case of two human bladder cancer and two human mammary cancer cell lines used in our studies. The cells of Hu1703He were more sensitive to carboplatin and its derivatives than the HCV29T bladder cancer cell line. In the case of human breast cancer cell lines it appeared that cells of the MCF-7 line were sensitive to carboplatin and its derivatives, whereas the T47D cell line was resistant to the cytotoxic activity of these compounds.

In order to pre-select the model for *in vivo* studies, all compounds were tested for their antiproliferative activity against cells of six murine neoplastic cell lines. As shown in Table 2, only the compounds **1–3** revealed cytotoxicity against five out of six cell lines, with ID_{50} values varying from 5.4 to 108.0 μM. The cells of P388 leukemia appeared to be the most sensitive target since all compounds, including **4–9**, revealed cytotoxic activity against these cells (Table 2). The ID_{50} values for **4–9** were 67.4, 88.6, 88.6, 54.8, 53.0 and 49.3 μM, respectively. All compounds, including reference carboplatin, were negative against the B16F-10 mouse melanoma cell line.

These results indicate that, in the context of structure–activity relationships, we can divide the tested platinum complexes into two groups: (i) complexes containing ethylenediamine (**1–3**) and (ii) complexes containing 1-alkylimidazole (**4–9**) as the neutral ligands. Complexes of group (i) revealed higher antiproliferative activity than reference carboplatin against 12 out of 14 cell lines used. Complexes **4–9** revealed significantly lower cytotoxic activity than

1–3 and carboplatin. In general, the results obtained are in accordance with the commonly accepted opinion that the presence of neutral amine ligands with the NH group is required for the cytotoxic activity of platinum complexes.[3]

Analyzing the results in light of the role of anionic ligands in platinum complex cytotoxic activity, we do not observe any significant differences between the enantiomers. This may indicate that their reactions with chiral biological structures are not stereospecific enough to distinguish the chirality of the platinum substrate or that the reactive form of the platinum complexes does not contain any anionic ligand. We suppose that the form which immediately reacts with DNA may probably contain an anionic ligand; however, not in an originally dianionic shape but as monodentate (O–).

Conclusion

In our search for low toxicity platinum cytostatics, we examined nine newly synthesized dicarboxylate complexes that were very similar in their chemical, structural and kinetic properties to carboplatin.[4] We found that malatoplatinum(II) complexes may be considered as possible candidates for clinical application because of their low toxicity, good water solubility and weak reactivity against biological thiols. This lower reactivity seems to correlate with low toxicity.[3] We have confirmed that the cytotoxic effect of these compounds depends mainly on the kind of amine ligand. Complexes with the presence of primary amine (ethylenediamine) were more effective in their cytotoxic activity *in vitro* than complexes containing tertiary amine (1-alkylimidazole).

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